

The University of Chicago

VARIATIONS IN GAMETOCYTE PRO-
DUCTION IN AVIAN MALARIA

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

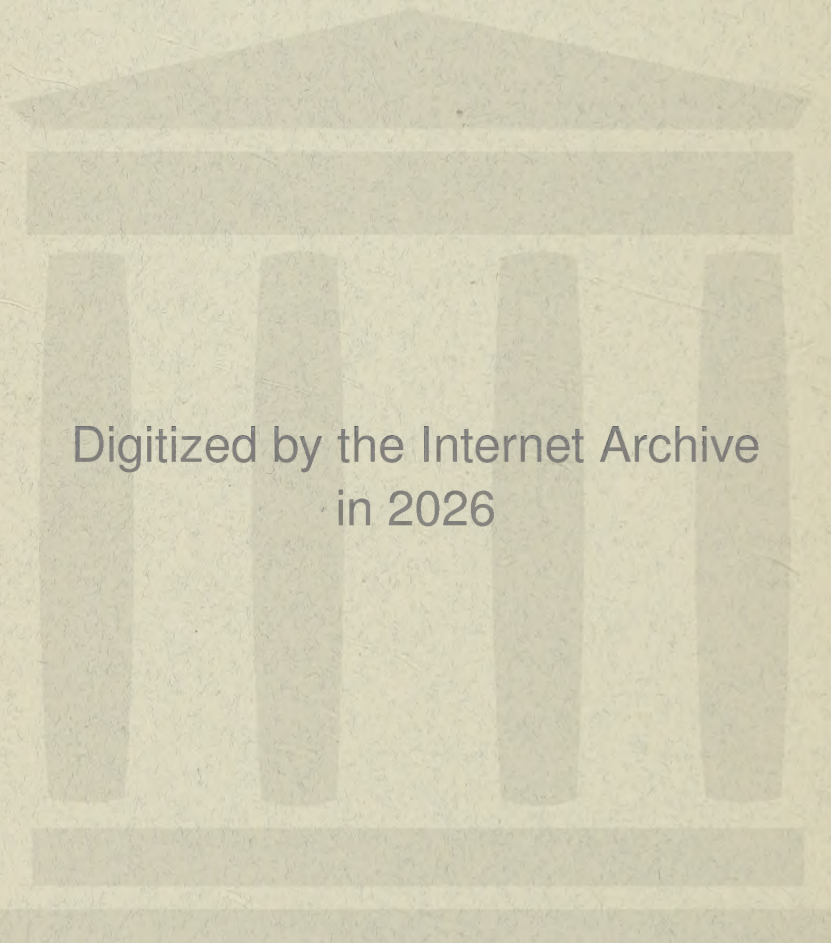
DEPARTMENT OF HYGIENE AND BACTERIOLOGY

1937

By
WINTON ELIZABETH GAMBRELL

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VARIATIONS IN GAMETOCYTE PRODUCTION IN AVIAN MALARIA¹

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NORMAL GAMETOGENESIS

The following study on avian malaria represents an attempt to amplify our knowledge of the origin and disappearance of gametocytes. The problem was attacked in two ways, by observations on the normal process of gametogenesis in strains having the full complement of gametocytes, and by contrasts with a strain which entirely lacks gametocytes.

Historical

Investigations on gametogenesis in bird malaria have been few and were not undertaken in the interest of the subject per se, but rather as outgrowths of other problems, particularly mosquito infectivity experiments. Neumann (1909) infected his canaries with 0.5 cc. of 0.85 per cent NaCl plus 0.5 cc. of infectious blood with an abundant number of parasites, "in order to obtain as large a number of gametocytes as possible." He observed from the seventh to ninth days severe attacks, with a large number of gametocytes in the blood findings. Taliaferro, L. G. (1925) found that the percentage of gametocytes in infections of *Plasmodium praecox*, during the acute period was around 3 per cent, with a marked increase of from 2 to 50 per cent during relapse and sometimes during the chronic period. She called attention to the difficulty of differentiating the full grown schizonts, just before division, from the macro-gametocytes. Huff (1927) found that

¹ This research was aided by a grant for Malaria Field Studies from the International Health Division of the Rockefeller Foundation.

the first gametocyte in *Plasmodium cathemerium* infections appeared four days after the first asexual forms, and that the peak for actual numbers of sexual forms came one day later than the peak for asexual forms. By counting only mature forms over a 24-hour period he concluded that the number of gametocytes showed no periodicity in distribution.

Shah (1934) reported that gametocytes in infections of *Plasmodium cathemerium* usually appeared in the peripheral circulation simultaneously with the asexual forms and that the peak in numbers corresponded with the asexual peak, and diminished correspondingly. He found a periodic development of gametocytes with maturation after about 24 hours corresponding with the sporulation of the asexual forms. Huff and Gambrell (1934) reported the first strain of avian malaria, *Plasmodium cathemerium* without gametocytes and called attention to the fact that some strains consistently showed a high number of gametocytes whereas others were poor in gametocytes. This present study is a continuation of that earlier work.

MATERIALS AND METHODS

The greater portion of this work has been done with strains of *Plasmodium cathemerium*, namely, the "D" strain (Huff, 1934) obtained from Rome and a strain derived from this, designated the "F" strain, which has no gametocytes (Huff and Gambrell). The original Hartman strain (Hartman, 1927), called the "H" strain, was used in some of the experiments, also the "N" strain which was derived from this through single cell isolation by Mr. Leslie Stauber. For contrast, *Plasmodium relictum* var *matutum* (Huff 1937) designated the "R" strain was studied in respect to gametogenesis because of its difference in segmentation time and its high percentage of gametocytes. The vertebrate hosts used throughout the experiments were female canaries, and in one particular study, English sparrows. The mosquitoes used were *Culex pipiens*, which had been bred in this laboratory for many generations and were quite susceptible, and *Culex fatigans* obtained from eggs and larvae in Georgia and also bred in this laboratory.

The birds were usually infected by intravenous inoculations of blood from infected birds by the technique employed in this laboratory (Taliaferro and Taliaferro, 1929; Gingrich, 1932).

Mosquito transmissions were effected by allowing stock mosquitoes to feed on infected birds following the method devised by Huff (1927). After two weeks or more the infected mosquitoes were exposed to an uninfected canary. If they failed to bite sporozoites were obtained in the manner first utilized by the Sergeants (1922). The mosquitoes were etherized, the thorax was severed from the abdomen, the wings and legs pulled off, and the remains of the thorax ground in physiological saline. The suspension with a minimum amount of debris was injected intravenously into birds.

When a highly synchronous cycle was desired, or when it was necessary to change or disturb the synchronism, the birds were placed in an electrically controlled light and dark cabinet designed and constructed by Mr. Stauber. Blood films were made in the usual manner, and were stained with a modification of MacNeal's tetrachrome stain (Gingrich, 1932). The number of parasites and of gametocytes have been given as the number per 10,000 red blood cells, in accordance with the procedure adopted by many workers on avian malaria. The probable error for observed values is as follows:

<i>Parasites per 10,000 erythrocytes</i>	<i>Per cent</i>
More than 150	10 or less
50-150	15 or less
Below 50	20 or less

Cytological characteristics of gametocytes

Before reporting the subsequent experiments, it seems advisable to give a full description of the gametocytes of the strains used and to establish criteria for identifying mature gametocytes and pregametocytes.

When stained with McNeal's tetrachrome stain, the gametocytes assume a characteristic uniform appearance. The macrogametocyte of *Plasmodium cathemerium* appears spherical with a large, rose-colored nucleus surrounded by cytoplasm which is a

deeper blue than the cytoplasm of the asexual forms. The nucleus itself seems composed of slender bacilliform structures rather than a solid mass of chromatin. At one side appears a distinct spherical granule which stains a deeper red. This granule is probably the karyosome. Wenyon (1926) described a similar structure in the macrogametocyte of *Plasmodium vivax*, but there has been no previous report of such a structure for any species of avian Plasmodia. The pigment is coarse, rod shaped and quite diffuse, having its natural deep brown color.

The microgametocyte is also spherical, the nucleus occupying three-fourths of the cell. The chromatin seems loosely arranged in thread-like strands and assumes a rose color. No distinct karyosome was observed, though some portion of the nucleus shows a deeper tint. In another strain of *relictum* not used in this study, this granule was found in microgametocytes also. The cytoplasm is pale pink in color, and over the whole cell are particles of coarse, brown, rod-shaped pigment.

The question of what constitutes a mature gametocyte has confronted many workers. The generally accepted criterion for maturity is the scattering of the pigment. From time to time others (Taliaferro, L. G., 1925; Huff, 1927; Shah, 1934) have noted the difficulty of differentiating forms that look like immature gametocytes from large schizonts. These forms must be pregametocytes for, in synchronous infections when a schizont approaches the size of a gametocyte, one would expect the nucleus to be divided. Another reason for this belief is that in the atypical strain of *cathemerium* which does not produce gametocytes, these forms are not present. It is relatively easy to find pre-macrogametocytes by means of the karyosome. These can be detected early, but this distinguishing characteristic is usually lacking in pre-microgametocytes. The pregametocytes that can be distinguished as such are spherical, have a nucleus like the macrogametocytes with a karyosome, but the cytoplasm is a pale blue color, and the pigment is not scattered but assembled in a mass in a vacuole in the cytoplasm.

The gametocytes of the "R" strain in *Plasmodium relictum* differ from those of *Plasmodium cathemerium* in the following

essentials. The pigment is more spherical than rod shaped, but is quite coarse. The nucleus of the microgametocytes appears more compact. Pregametocytes cytologically resemble those of *Plasmodium cathemerium* but appear slightly larger.

Relation of gametogenesis to schizogony

a. *Numbers of gametocytes during a typical cycle.* If gametocytes arise from asexual forms as has been generally believed for some time, then the appearance of gametocytes should show some relation to the asexual cycle. The broods of maturing gametocytes should be obvious at definite time intervals. In order to see whether waves of gametocytes occur, three birds were injected intravenously with the "D" strain of *Plasmodium cathemerium* and three with the "R" strain of *Plasmodium relictum*. These two strains were chosen because they both have strict quotidian periodicity, produce ample numbers of gametocytes, and offer a natural contrast to each other, in that the "D" strain segments in the evening hours around 6 p.m. and the "R" strain segments in the morning hours around 8 a.m. The birds were placed in a light and dark cabinet on a schedule of 12 hours of darkness and 12 hours of light. Blood films were made at 3 hour intervals for 3 days. The parasites were counted and expressed as the number per 10,000 red blood cells and the gametocytes were counted separately in the same manner. Figure 1 represents the numbers of gametocytes in typical cycles in the two types of infection.

There was a definite increase in numbers of gametocytes around the time of segmentation. Sometimes this occurred a little before, but usually several hours afterward. It must be borne in mind that the gametocytes which are recognizable as such did not arise at the segmentation peak at which they become recognizable, but at a previous segmentation time, possibly the one 24 hours earlier. These counts also revealed that the actual numbers of gametocytes varied greatly at different hours during the day and night. The increase was not due so much to a cumulative process but rather to additions of fresh broods at definite time intervals.

In view of these findings it seemed expedient to obtain counts

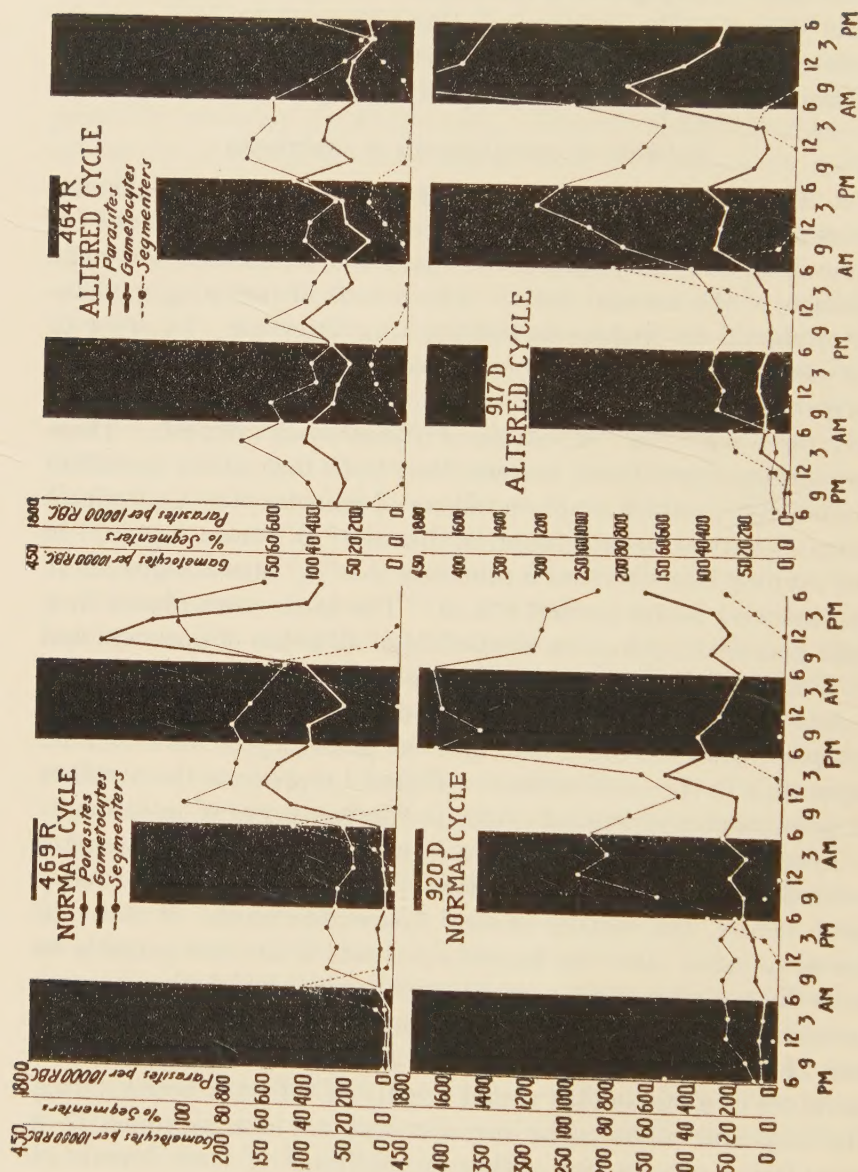


FIG. 1

placed on a similar schedule, 12 hours of light and 12 hours of darkness. Blood films were taken during the entire course of infection, a period of 10 days. The times for making observations were those periods just prior to and just after segmentation; for the "D" birds, 12 m. and 3, 6, 9, and 12 p.m., and for the "R" birds 12 p.m., 6 and 9 a.m., and 12m. Figure 2 shows a representative infection of each case. With the increase in asexual forms, there was also an increase in sexual forms. In the *relictum* infections of this particular strain, the gametocytes were swept out when the immunity to the asexual forms was established; in the *cathemerium* infections, the gametocytes persisted but the numbers tended to decrease as the asexual forms decreased.

b. *Effect of changes in the segmentation time on gametocyte production.* If the production and maturation of gametocytes are actually correlated with the asexual cycle, then any change of the cycle should show a similar change in the production of gametocytes.

Seven birds were subjected to an altered cycle, 4 infected with the "D" strain of *Plasmodium cathemerium* and 3 with the "R" strain of *Plasmodium relictum*. These birds were kept in the light and dark cabinet, with the usual daylight period changed to darkness at 12-hour intervals, for 2 weeks before being inoculated. The birds from which these were inoculated were kept on the same schedule a sufficient time to shift the segmentation time so that it occurred 12 hours later than usual, before the inoculation was made into the experimental birds. Slides were made at 3-hour intervals for 3 days. Figure 1 shows the trends of parasite and gametocyte numbers.

The peaks of gametocyte numbers in the altered *cathemerium* infections followed the peaks of asexual forms, and occurred after segmentation. When the "R" strain was put on this schedule, its segmentation peaks tended to flatten out, the periodicity was the same but the synchronism was altered. The curve suggested incomplete alteration in the cycle, and the peak of segmentation occurred earlier than was expected, -3 p.m. instead of 8 p.m. There was a corresponding alteration in the peaks of gametocyte numbers.

c. *Disturbances in synchronism and their effect on gametocyte production.* As a result of the findings that a change in the time of segmentation of the asexual parasites artificially induced by altering the schedule of light and darkness led to a corresponding change in the time of the appearance of pre-gametocytes, an attempt was made to disturb the synchronism of the "D" strain of *cathemerium* and the "R" strain of *relictum* so that asexual forms would reproduce at irregular intervals. The rationale of this experiment was based on two previous observations; first, that the strains of *cathemerium* in this laboratory which lack gametocytes have also lost the synchronism of the asexual cycle, and second, that in Boyd's (1929b) report the infection in the experimental bird subjected to 24 hours of light and 24 hours of darkness presented an asexual cycle very similar to that of the "F" strain of *cathemerium* which has no gametocytes. Five birds infected with the "D", "R", and "F" strains were subjected to 24-hour periods of light and dark, and three control birds were allowed to remain on the usual 12 hour cycle. The "F" strain was used for comparison with the other two, particularly as to changes in morphology of the parasites. Blood films were made at 6-hour intervals for the duration of the infection. Birds inoculated with the "R" strain did not show a patent infection long enough to reveal any disturbance in the cycle. The infections of the "D" strain showed a decided fluctuation in the multiplication of the asexual forms and the occurrence of gametocytes but no decrease in the numbers produced. There was no change in the morphology of the parasites. Subinoculations from these birds resulted in infections with the usual number of gametocytes. There appeared to be no impairment in the ability of the parasites to produce gametocytes when the synchronism was disturbed by prolonged periods of light and dark, but an irregular schedule of gametocyte production resulted.

Population studies during the course of the cycle

The preceding experiments from data obtained by enumerative methods indicated that the gametocyte population was augmented periodically at approximately the same time as were the

asexual parasites. A tabulation of the different components of parasite populations during a 24-hour period was made in order to ascertain the following points: (a) whether new broods of

TABLE 1

Ratio of mature and immature gametocytes and ♀ and ♂ gametocytes over a 24-hour period at 3-hour intervals

TIME	PLASMODIUM CATHEMERIUM															
	Normal schedule								Reversed schedule							
	Bird 920 D				Bird 921 D				Bird 916 D				Bird 917 D			
	♀	♂	Pre	To- tal	♀	♂	Pre	To- tal	♀	♂	Pre	To- tal	♀	♂	Pre	To- tal
12 m.	37	14	0	51	38	11	0	49	47	16	0	63	26	19	3	48
3 p.m.	34	5	10	49	24	12	18	54	24	21	0	45	29	19	0	48
*6 p.m.	30	2	19	51	23	10	14	47	25	20	0	45	33	20	0	53
9 p.m.	26	10	12	48	23	10	15	48	33	20	0	53	30	20	0	50
12 p.m.	44	18	12	74	31	8	12	51	36	10	0	46	25	15	5	45
3 a.m.	28	20	0	48	32	9	4	45	15	10	27	52	16	10	25	51
6 a.m.	32	15	0	47	37	12	3	52	14	4	31	49	12	3	30	45
†9 a.m.	34	18	0	52	36	13	1	50	13	4	40	57	23	12	16	51
12 m.	37	8	0	45	36	16	4	56	49	12	0	61	37	15	4	56

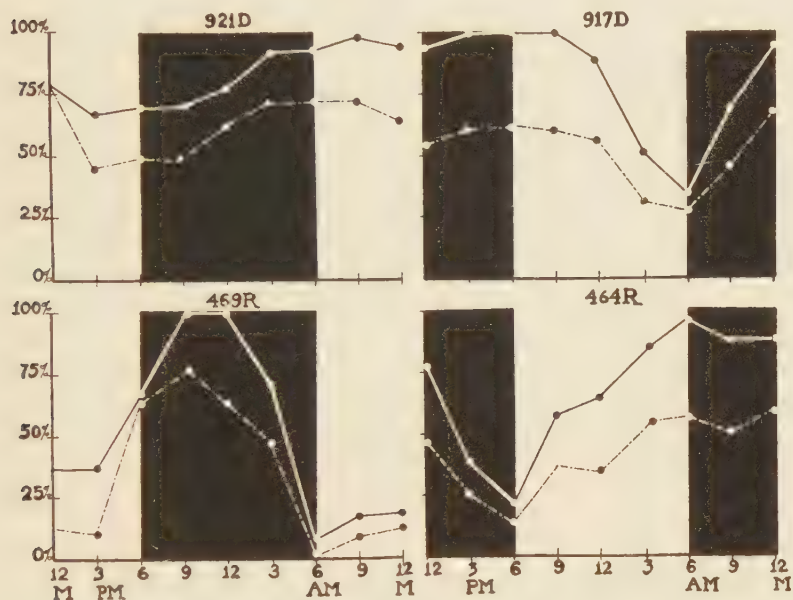
TIME	PLASMODIUM RELICTUM VAR. MATUTINUM															
	Normal schedule								Reversed schedule							
	Bird 468 R				Bird 469 R				Bird 463 R				Bird 464 R			
	♀	♂	Pre	To- tal	♀	♂	Pre	To- tal	♀	♂	Pre	To- tal	♀	♂	Pre	To- tal
12 m.	8	0	40	48	6	12	31	49	25	19	0	44	25	17	11	53
3 p.m.	36	8	14	58	5	14	33	52	11	8	28	47	12	6	30	48
†6 p.m.	37	16	4	57	29	3	16	48	7	3	49	59	7	4	44	55
9 p.m.	28	27	0	55	37	11	0	48	12	11	40	63	18	11	21	50
12 p.m.	28	18	5	51	33	19	0	52	8	11	35	54	17	15	19	51
3 a.m.	28	20	7	55	21	11	13	45	9	21	23	53	26	15	7	48
6 a.m.	15	3	40	58	0	4	50	54	26	20	0	46	28	20	2	50
*9 a.m.	14	3	36	53	5	5	54	64	38	16	0	54	23	17	6	46
12 m.	8	3	41	52	6	3	45	54	25	23	0	48	30	15	7	52

* Segmentation time on usual schedule of light and darkness.

† Segmentation time on reverse schedule of light and darkness.

gametocytes (pregametocytes) were more prevalent at definite times, (b) at what time new broods became mature, and (c) in what ratio the macro-, micro-, and pre-gametocytes occurred.

Blood films previously employed in the study of the cycle were utilized in this study, and a careful analysis made on eight birds, 4 on the normal cycle, and 4 on the reversed cycle. The 24-hour period during the acute rise was studied. Table 1 and figure 3 summarize the results.



PERCENTAGE OF GAMETOCYTES IN *P. cathemerium* (D) AND
P. relictum VAR. *matutinum* (R)

—●— % MATURE GAMETOCYTES - - -●- - % MACROGAMETOCYTES

FIG. 3

In these two types of infection which undergo a schizogonous cycle every 24 hours, the gametocytes also show periodic changes. Potentially sexual parasites are clearly distinguishable when the asexual forms begin dividing, usually 6 or 8 hours before segmentation is complete. They gradually assume the characteristics of mature macro- and microgametocytes, requiring a period from 6 to 10 hours after segmentation of the asexual forms to

accomplish this. The procedure takes place no matter what the hour of segmentation is, so long as the cycle is kept synchronous. Therefore, assuming that the potential gametocytes originate from merozoites, the time required for their maturation is approximately 6 or 8 hours longer than the time required for an asexual form arising at the same time to complete its cycle.

At the time when the pre-gametocytes are most prevalent, they all appear to be pre-macrogametocytes, the ratio of macro- to microgametocytes varying from 50:1 to 5:1. These forms which seem to be pre-macrogametocytes are possibly undifferentiated cells which form either macro- or microgametocytes, for at maturity a few hours later the ratio of the two sexes is more nearly equal, though there is usually a slight preponderance of macrogametocytes. The ratio of the two sexes seems to vary at different hours. The numbers counted are small and are suggestive rather than proof of this point.

The numbers of merozoites present in the mature segmenters of the "D" strain of *cathemerium* were determined. The standard deviation was derived by the formula

$$\sigma = \sqrt{\frac{\sum x^2}{n} - M^2}$$

and the probable error calculated from this (x_1 , Pearson's tables). The mean number of merozoites for this strain in 1935 was $12.95 \pm .17$, and in 1936 was $13.72 \pm .16$. The difference, $.77 \pm .24$ is not clearly significant.

From these studies over a period of 24 hours, constant changes appear to take place. The sexual elements change from immature to mature forms and the ratio of the two sexes when mature appears to vary at different hours. The time when a blood film is made may influence greatly the conclusions as to the percentage of gametocytes present and the ratio of macrogametocytes to microgametocytes.

Initial occurrence of gametocytes

a. *Subinoculations of whole blood by intravenous and intramuscular injections.* When inocula from patent infections were used

the resulting infections occurred immediately and gametocytes were present, being carried over from the donor. The numbers of gametocytes increased as the asexual forms increased and after the acute rise usually decreased in numbers, being swept out of the peripheral blood along with the asexual forms.

Although most of the infections were passed from bird to bird while still patent, quite a number of injections were made from birds with latent infections when no parasites could be detected in the inoculum. The manner of inoculation, whether intravenous or intramuscular seemed to make little difference in the time elapsing between the injection and the appearance of parasites. Usually 5 days passed before any parasite could be found and quite often this period extended to 6 or 7 days. In the majority of cases, if gametocytes appeared at all, they appeared simultaneously with the trophozoites. In a small number of cases they were found one day later than the asexual forms.

b. Intravenous injection of whole blood which had stood at room temperature. An attempt was made to obtain an inoculum free of gametocytes from a gametocyte-producing strain in order to determine just how long it took the potential gametocytes to develop. Theoretically, this should be possible by two methods; first, by allowing the infected blood to stand after withdrawal from the host until exflagellation of the microgametocytes and fertilization of the macrogametocytes has occurred, or second, by allowing this same process to take place in the stomachs of mosquitoes fed on infected birds. Both of these methods were tried, but an inoculum entirely free of gametocytes was never obtained. The most successful approach was made by allowing infected blood to stand at room temperature in heparinized physiological saline solution. Although gametocytes remained in the inoculum an accurate count on the numbers present per 10,000 erythrocytes was obtained as well as a similar count on the parasites present in the inoculated birds immediately after injection. With this information, the rise in gametocytes could be detected and the time of occurrence noted. Bird 915D, with a count of 2264 parasites, 114 gametocytes per 10,000 red blood cells, was used as donor. The blood was collected from the jugular vein in physio-

logical saline containing heparin, and allowed to stand at room temperature for two hours. Two blood films were made from this suspension and the number of gametocytes noted, then 0.5 cc. of this suspension was injected intravenously into two birds,

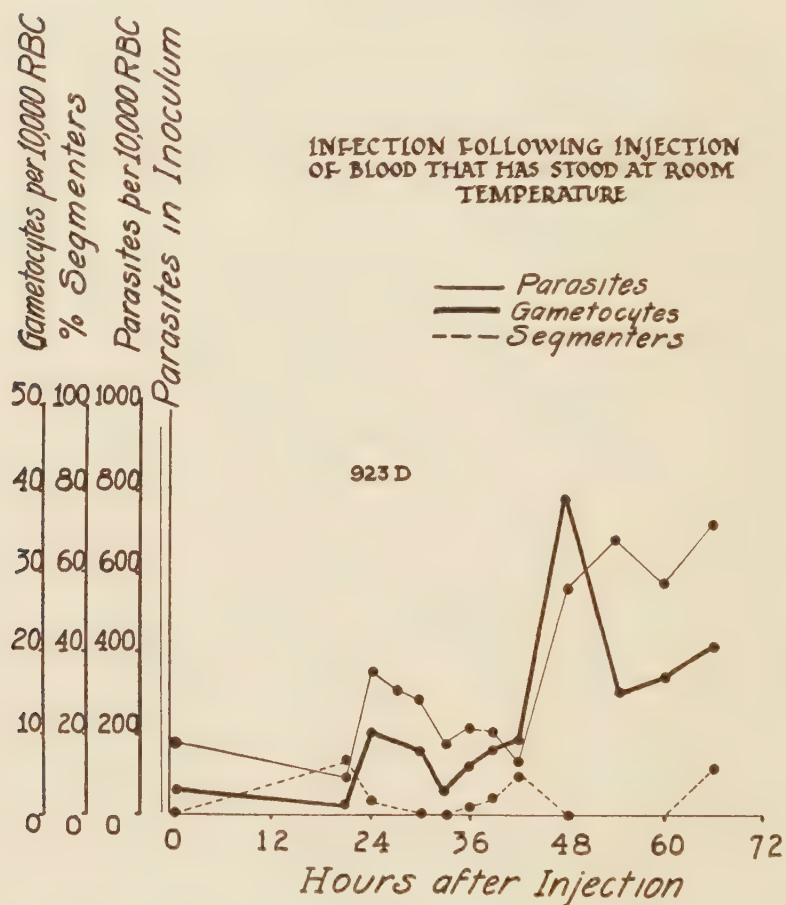


FIG. 4

923D and 924D. Two minutes later, these birds were examined for gametocytes in the peripheral blood. No further counts were made until 21 hours had elapsed, then films were examined at 3-hour intervals. At the time when the asexual forms of the para-

sites were completely segmented there was a concomitant increase in the sexual forms. (See fig. 4.)

c. Intravenous inoculation of blood meals from mosquitoes. Blood meals from engorged mosquitoes were injected into birds. The abdomens of 28 mosquitoes which had fed 3 hours previously on an infected bird whose blood contained numerous gametocytes, were ground in physiological saline and injected intravenously into two clean birds. The abdomens of 14 engorged mosquitoes treated in the same manner after the same time interval were injected into two other birds. In these cases the numbers of

TABLE 2

NUMBER OF BIRDS INJECTED IN- TRAVENOUSLY WITH SPOROZOITES	DAYS ELAPSING BEFORE PARASITES APPEARED	DAYS ELAPSING BEFORE GAMETOCYTES APPEARED
1	5	5
1	5	6
1	6	6
1	6	7
1	6	9
1	6	10
4	8	8
2	8	9
1	9	9
2	Negative	
Total injected.....		15
Number infected		13

parasites injected were small and the infection did not become apparent until 48 hours later. After extensive search an occasional gametocyte was observed on the third day but the infection was far too low to get significant counts. During the period when segmenting forms of the asexual parasites were most prevalent, one bird (926D) which had received the largest inoculum showed an increase from 30 to 223 asexual parasites and from 5 to 20 gametocytes per 10,000 red blood cells. A similar increase was noted on the fourth day in the other birds.

These two sets of experiments add confirmatory evidence that forms recognizable as gametocytes require at least 24 hours to

develop and that they consequently increase in numbers around the time of complete segmentation of the asexual forms.

d. Intravenous injection of sporozoites from mosquitoes. Sometimes in attempting to transmit infections from mosquitoes to birds, the mosquitoes failed to bite. This necessitated grinding the head and thorax of the mosquitoes in physiological saline and injecting this suspension of sporozoites intravenously into the birds. In a recent research note Wolfson (1936) called attention to the fact that all previous workers have been unsuccessful in infecting canaries by intravenous injection of sporozoites. She reported the successful infection of two birds by this method after an incubation period of 4 weeks. In view of previous findings the results in table 2 seem worthy of inclusion. Two other birds were injected with saline suspensions of infected mosquito stomachs. Sporozoites were present, but failed to infect the birds.

Effect of long continued passage by subinoculation on gametocyte production

When patent infections are passed for a long period by subinoculation from bird to bird without allowing the parasites to assume a latent period, the numbers of gametocytes invariably decrease, but only in two cases have they entirely disappeared. The decrease is usually gradual. When an infection which originally showed a large number of gametocytes is allowed to undergo a period of latency extending from a few weeks to several months or years, subsequent inocula obtained from this latent infection produce infections with equally large or even larger numbers of gametocytes. In the course of these studies no strain has retained its maximum capacity of gametocyte production after long continued passage. Some strains which had once yielded a population of 50 per cent gametocytes showed a decrease to 0.1 per cent gametocytes or one gametocyte in every thousand parasites after being passed from bird to bird for a month. There seems to be a great diversity in the ability of strains to produce gametocytes. Some branches of the "D" strain are poor producers of gametocytes, consistently showing only a few. No seasonable variation was noted, nor does the age of the host

appear to have any influence on the numbers of gametocytes produced. This decrease of gametocytes was treated in detail in an earlier report (Huff and Gambrell) and subsequent studies involving several hundred additional birds confirm these results.

Effect of mosquito transmission on gametocyte production

Infections resulting from mosquito transmission are of considerable interest, particularly in regard to the ability of the parasites to form gametocytes because of the possibility that sexual multiplication may alter this function. At a time when a highly susceptible strain of *Culex pipiens* was available for transmission experiments, an attempt was made to pass infections from mosquito to bird, and back to mosquito, thereby maintaining a continuous chain of infections in the natural manner as long as possible. This was possible for only three complete generations; three transmissions to mosquitoes and three transmissions from them to clean birds, and was interrupted at the end of three months. The series was begun by grinding a mosquito infected with the "H" strain of *catheimerium* in physiological saline and injecting it intravenously into bird 1012H. The maximum number of parasites in the resulting infection was 2,880 per 10,000 red blood cells, 10 per cent of these being gametocytes. One mosquito fed on this bird and was injected into two birds (1022H and 1023H) 15 days later. Only one of these developed an infection (1023H), showing a maximum of 131 parasites per 10,000 red blood cells, 5 per cent of these being gametocytes. Two lots of mosquitoes were fed on this bird, three of these mosquitoes were dissected, and found to be infected with 16 to 28 oöcysts. Two of these mosquitoes were ground and injected into three birds (1029H, 1030H, and 1031H). All developed infections, but none of these showed a higher infection or a higher percentage of gametocytes than the first bird in this series. Single transmissions were obtained on 10 other occasions and in no instance was there a perceptible increase in the ability of the parasites to produce gametocytes, after passing through a mosquito. I was not successful in transmitting by mosquitoes a strain which regularly produced only a few gametocytes. Mosquito transmission in

those cases which were successfully transferred apparently does not enhance the ability of certain strains to produce gametocytes, nor does it decrease the percentage ordinarily produced by the strains.

STUDIES ON AN ATYPICAL (GAMETOCYTELESS) STRAIN

Origin of the strains

The history of the primary strain of *Plasmodium cathemerium* used in these experiments has been given in part in two earlier papers by Huff (1934) and Huff and Gambrell (1934). This atypical strain designated the "F" strain was regarded as an excellent control on experiments concerning the normal process of gametogenesis. Because of the absence of gametocytes, the "F" strain was regarded as a biological anomaly until another assumed atypical characteristics. During the summer of 1935 one branch of the "N" strain which originated from single cell isolation by Mr. Stauber, developed similar deviations from the parent strain. In the course of experiments which entailed alterations in the synchronism of the asexual cycle, the parasites in one bird (252N) failed to produce gametocytes. The host had not been subjected to any experimental procedures but had received the inoculum from a bird in which the course of infection had been altered by light and dark experimentation. The parasites had been passed from the experimental bird (354N) to two others, (249N and 250N and from 249N to 252N). Usually the cycle of the infection returns to its quotidian periodicity but it failed to do so in this case, and actual observation of 1000 parasites revealed no gametocytes. Three more birds which had been injected from the same source were also without gametocytes. Injections were made from them, but the birds receiving inocula died very quickly, apparently from some cause other than the malarial infection, for death usually occurred before the parasites increased enough in numbers to be fatal. Out of 19 birds infected, only 6 recovered from the acute stage, and only 2 of these survived for 4 months. Attempts were made to isolate bacteria, since the pathology suggested that a concomitant bacterial or virus infection was being passed with the parasite. Blood cultures yielded

a heterogenous group of organisms and the work had to be terminated before the etiology could be established definitely. Four months later, inoculations were made from the 2 surviving birds and the ensuing infections still showed no gametocytes. This strain has not been studied so extensively as the "F" strain, but it has been maintained up to the present time, and in a recent count of 10,000 parasites no gametocytes were found. This seems to indicate that loss of gametocytes is not such a rarity in nature as was thought at first.

Characteristics of the atypical strains

At the time the first atypical strain arose, certain outstanding differences were noted between it and the original strain. The most striking characteristic was the loss of gametocytes. At the same time a distinct loss of synchronism occurred in the asexual reproductive cycle. Taliaferro (1925), Hartman (1927), and Boyd (1929a) have demonstrated the strict quotidian periodicity of *Plasmodium cathemerium*. The original "D" strain conformed in every way to their findings, but in the "F" strain reproduction apparently was taking place at all hours of the day for subsequent tests for synchronism revealed from 2 to 10 per cent segmenting forms present in every blood film. Later, when the "N" strain assumed atypical characteristics, the disturbed synchronicism was the first thing noticed and led to a check on gametocyte production. In this case, the synchronism of the infection occurring in the original bird had been disturbed purposely, but other birds in the same series had undergone identical treatment, and their infections had shown no impairment in the function of gametocyte production. Also, in due course, their synchronism returned to normal and birds inoculated from them showed typical *cathemerium* infections whereas inoculations from 252N gave rise to infections with disturbed synchronism long after the time usually required for it to return to normal.

The reproductive activity of the "F" strain has been checked at intervals over a period of three years. Figure 5 shows the cyclic tendencies of this strain. The birds used in testing for periodicity were kept in the light and dark cabinet on a syn-

chronized cycle of 12 hours of light and 12 hours of darkness. At present it appears to be approaching a quotidian periodicity, although the highest percentage of segmenting forms found was 18. These forms are more abundant at 24-hour intervals, but segmenting forms are still present at all hours.

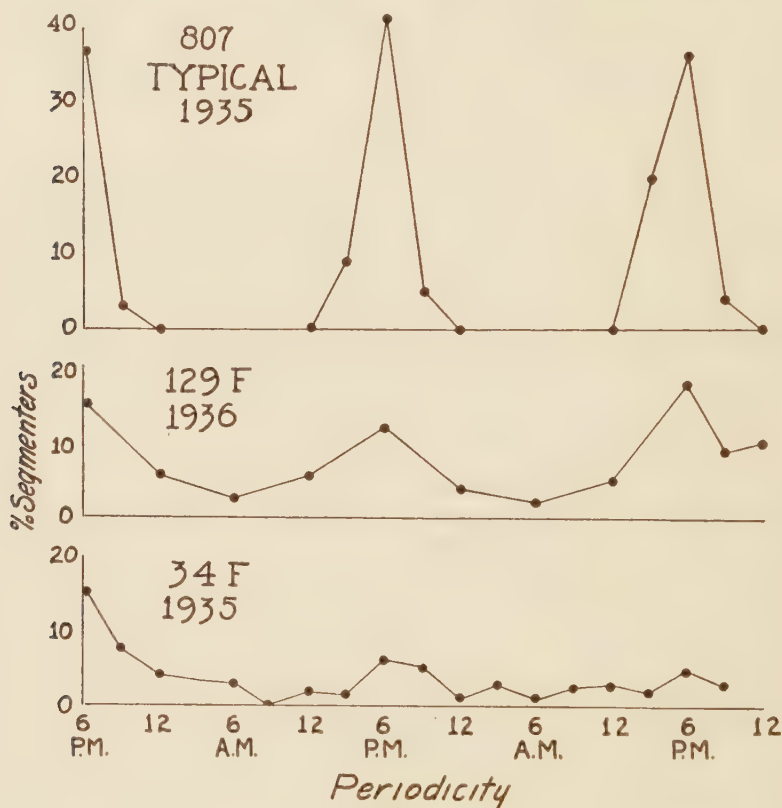


FIG. 5

Morphologically, the "F" strain differs in other respects from the parent strain. The parasites themselves are not so distinct in their outlines but assume a "ragged" appearance, and when stained seem to absorb less of the dye since they appear slightly paler in color. The number of merozoites in the segmenting forms was compared with the number in the "D" strain. See

table 3. The "F" strain when compared with the "D" strain shows a difference of from $3.66 \pm .21$ to $5.94 \pm .18$ merozoites per segmenter or an average of $4.95 \pm .14$ which is significant. De Buck (1935) found a similar difference in a Holland strain and a Madagascar strain of *Plasmodium vivax* and showed that it was a real difference in the two strains irrespective of whether they were transferred by mosquitoes or by blood inoculations.

The sparrow as host to the atypical strain

In order to see whether transferring the atypical strain back to its original host, the sparrow, would have any effect on the

TABLE 3

BIRD	SEGMENTERS COUNTED	MEAN NUMBER MEROZOITES	DIFFERENCE	
		<i>P.E.M.</i>		
36F (1935)	100	$8.99 \pm .14$	36F and 161F	$1.21 \pm .16$
161F (1936)	101	$7.78 \pm .07$	807D and 920D	$.77 \pm .24$
807D (1935)	105	$12.95 \pm .17$	807D and 36F	$3.66 \pm .21$
920D (1936)	104	$13.72 \pm .16$	920D and 161F	$5.94 \pm .18$
			807D and 161F	$5.25 \pm .19$
"D" with "F" difference.....		$4.95 \pm .14$	920D and 36	$4.73 \pm .68$

parasite, young English sparrows were trapped and tested for infection by examination of blood films, and by subinoculations into canaries. Twelve young sparrows were obtained free from infection. Five of these were inoculated with blood from a bird having a patent atypical infection and there developed similar infections. No gametocytes appeared. Transfers of the infection from sparrow to sparrow were not made because of the high mortality and injury of the captive birds. However, one sparrow (136F) had an infection lasting for 30 days and terminating fatally. Although parasites were quite numerous during this infection, no gametocytes were found. The atypical strain remained atypical in all of its characteristics, in spite of being in its natural host.

Attempts to infect mosquitoes of known susceptibility

The inability of one strain of *Plasmodium cathemerium* to infect highly susceptible *Culex pipiens* mosquitoes became evident as a result of mosquito infectivity experiments conducted over a period of years by Huff (1927, 1929, 1930, 1931, 1932, 1934). The emphasis at this time was on the reaction of the mosquitoes rather than the Plasmodia, as only highly infectious strains of malaria were desirable in testing mosquitoes for immunity. Routine examinations of blood films from birds with patent infections of the "D" strain revealed that the number of gametocytes present

TABLE 4

LOT	PARASITES PER 10,000 RED BLOOD CELLS	GAMETOCYTES PER 10,000 RED BLOOD CELLS	NUMBER DISSECTED	NUMBER POSITIVE
3	1,900	0	3	0
5	1,530	0	9	0
9	1,230	0	10	0
10	1,920	0	8	0
12	530	0	12	0
			42	0
Controls fed at approximately the same time				
6	1,313	65	2	2
7	1,277	217	1	1
8	306	6	5	4
			8	7

was far below the criterion chosen for successful mosquito infectivity experiments. Further studies showed that this strain was practically without gametocytes and unsuitable for use in testing mosquito immunity, but was of interest in that it had formerly produced tremendous numbers of gametocytes and was evidently losing this ability of gametogenesis. Microscopic examination of a blood film for gametocytes at best offers only a limited field, and is time consuming. A highly susceptible strain of mosquitoes offers a delicate means of testing whether gametocytes which are not evident by the usual methods of examination may yet be

present in sufficient numbers to cause the formation of an oöcyst. Shah, Roseboom and Del Rosario (1934) showed that the average weight of the blood meal ingested by *Culex pipiens* was 1.2 mgm. The volume of blood ingested offers a reasonable expectation that infections might result from the presence of a very small number of gametocytes. These same authors found that only one out of 11 mosquitoes became infected when fed on blood meals in which no gametocytes per 500 leukocytes were found.

Susceptible mosquitoes were fed on birds infected with the "F" strain of *catheherium* and dissected after a period of 5 to 7 days. The stomachs were examined for oöcysts in the usual manner by wet preparations. Table 4 gives the results of these experiments.

Culex fatigans was later used in the feeding experiments after the strain of *Culex pipiens* had been lost. These mosquitoes took blood meals readily; often the average number of mosquitoes engorged from a single bird was 45 and sometimes as many as 75 or 100. Dissections of these mosquitoes showed the absence of oöcysts, but the importance of these findings is lessened by the fact that the controls which were fed on birds infected with a large number of gametocytes showed a low number of oöcysts and often no infections.

Attempts to transfer by mosquitoes

Some of the mosquitoes from each of the lots recorded above were not dissected but were saved for feeding experiments. Additional lots were fed on birds infected with the "F" strain over a period of three years and transfers attempted. The mosquitoes were kept for two weeks after they had taken a blood meal then allowed to bite uninfected birds. Only one lot which had fed on the "F" strain ever took a second blood meal and the bird which was bitten by these mosquitoes failed to develop an infection. Attempts were made to obtain infections by grinding the head and thorax of the mosquitoes in a small amount of 0.85 per cent NaCl and injecting this suspension into birds. Repeated attempts failed at all times to bring about an infection with mosquitoes fed on the "F" strain, whereas this method was successful in 13 out of 15 trials with mosquitoes which had fed on birds with infections showing gametocytes.

These negative results, together with the absence of oöcysts in highly susceptible *Culex pipiens* mosquitoes, indicated that the "F" strain lacked gametocytes, or that if they were present, they were too few in number to cause an infection in susceptible mosquitoes.

Compatibility of typical and atypical strains of same species

a. Cross-immunity tests. The reactions of the atypical strain with the typical strain in cross-immunity tests were investigated as soon as its difference from the parent strain were noted. The possibility that the typical strain of *catheimerium* had become mixed with another species which showed differences of periodicity, staining reaction and pathogenicity was recognized, and although this possibility seemed remote, every known means of determining the species were utilized. By cross immunity tests similar to those described by Gingrich (1932), it was shown that complete cross immunity existed between the two strains. These tests were interesting in that by injecting the "D" strain showing gametocytes into birds which had recovered from the "F" strain, a means was available for the first time for detecting how long the super-imposed infection remained in the immune bird. Sub-inoculations from the superinfected bird into birds at definite time intervals resulted in infections with gametocytes so long as the super-imposed strain was still present in the immune bird. In one case the super-imposed strain persisted along with the original infection in the immune bird for 8 months. In all other cases it disappeared in less than 4 months. The ability to remove gametocytes was just as great in birds which had had infections with no gametocytes as in those which had developed typical infections with gametocytes. In all superinfected birds, whether they had been infected first with the typical or atypical strain, the gametocytes of the super-imposed strain were not removed as readily as the asexual forms, and persisted in the peripheral circulation longer than the asexual forms.

b. Effect of simultaneous infections with the two strains. The "F" strain seemed to have a more pathogenic effect on infected birds. Therefore, a deliberate attempt was made to find out

what effect mixed infections of the typical and atypical strains would have. Since the "F" strain lacked gametocytes it was felt that it might be inimical to a strain with gametocytes, although the cross-immunity tests reported above in which the typical strain had remained for 8 months in a superinfected bird formerly infected with the atypical strain offered evidence which might be interpreted as contrary.

Mixed infections of the two strains were carefully studied in 10 birds. Inocula containing approximately the same number of parasites of the "F" and "D" strains were obtained by mixing whole blood from birds with patent infections of these respective strains. In no case, did gametocytes fail to develop in the infected birds. The percentage of gametocytes was lower in most instances but the gametocytes which developed were normal in appearance. Subinoculations from birds which had recovered from mixed infections gave rise to infections which had typical gametocytes. The "F" strain apparently lacked the power to supplant completely the "D" strain, or affect its ability to produce gametocytes. If the absence of gametocytes in this strain had been caused originally by some deleterious agent, these results indicate that this agent was no longer present, or able to affect another strain.

c. Effect of serum from birds with atypical parasites on infections with typical parasites. Early in these studies, the possibility that the loss of gametocytes in the "F" strain was due to some inimical factor in the blood serum of the host was investigated. Combinations of serum from birds which had infections with no gametocytes, and parasitized cells washed free of serum from birds having the typical infections were made. Also serum from birds with typical infections was combined with parasitized cells from birds with gametocyteless infections. Controls were run on the whole blood and on the cell free serum. In the first experiment, the serum and cells were obtained by collecting the whole blood in a small amount of citrate solution. The blood of three birds infected with each strain was pooled. Injections of whole blood were made into two clean birds and the remainder was centrifuged for an hour at high speed to pack the cells and leave the serum

clear. The serum was then removed with a capillary pipette and placed in a sterile tube. To free the serum from parasites, it was filtered through a small Seitz filter. The cells were washed three times in 0.85 per cent NaCl by centrifugation, the saline was then decanted and the cells combined with the serum. By using this number of birds enough serum and cells could be obtained each time to mix with the washed cells later, and also have a sufficient amount left to inject into the controls. Approximately 0.4 cc. of each combination of cells and serum was injected into two birds each. These combinations were made with the following results:

"D" serum + "F" cells.....	No gametocytes
"F" serum + "D" cells.....	Many gametocytes
"D" serum.....	No infection
"F" serum.....	No infection
"D" whole blood.....	Many gametocytes
"F" whole blood.....	No gametocytes

The serum from birds with the atypical strain did not inhibit the production of gametocytes, nor did the serum from birds with the typical strain stimulate the production of gametocytes. The ability or inability of the parasites to produce gametocytes seems to be independent of any factor in the serum.

Further studies on infected and super-infected birds

Two birds (291D and 441D) which had been among the first to show atypical infections in the earlier experiments of Huff and Gambrell (1934) were still alive at the end of 36 months and 42 months respectively after their initial infection. Clean birds were inoculated with blood from these birds to see whether the infection still remained in them, and whether the parasites had regained their ability to produce gametocytes in this long time interval. The resulting infections showed no change in the characteristics of the atypical infection. Prolonged search failed to reveal any gametocytes. After three years in one bird and three and one-half years in another, the infection still persisted and the parasites had not regained their ability to produce gametocytes.

Subinoculations were made from two birds (457D and 514D) which had been thoroughly studied in an extensive cross-immunity test in 1933 (Huff and Gambrell) and were still alive after three years. Bird 514D which had been infected first with the atypical strain and superinfected with the typical strain gave rise to infections showing no gametocytes and with the other characteristics of the infection which it had shown originally. Bird 457D which had been infected first with the typical strain and superinfected with the atypical gave rise to a series of infections with a high percentage of gametocytes, some of the birds subinoculated from it showing infections with 20 to 30 per cent gametocytes. In spite of an injection of a large inoculum of atypical parasites in the original superinfection experiment, the ability of this strain to produce gametocytes was not at all impaired. No means were available to tell whether the atypical strain was also present in this series, so subsequent transfers were made by mosquito passage to insure its absence. After a long time interval these super-infected birds gave rise to infections with all the characteristics of the strains they were infected with originally.

DISCUSSION

Throughout these investigations two theoretical questions have been constantly faced; first, what mechanism is responsible for gametocyte formation, and, second, what caused the disappearance of gametocytes from the strains which lack them. When the answer to the first question is thoroughly understood, doubtless, the explanation for the second will be obvious.

For some years there has been a growing tendency to assume that gametocytes arise from the asexual parasites in the course of the schizogonous cycle. Whether or not this ability to produce gametocytes is inherent in the parasite and functions irrespective of external stimuli; or whether it is a direct result of environmental influences is still a matter of conjecture. From investigations on human malaria the following ideas in regard to gametogenesis have been advanced. Mannaberg (1894) described the origin to the union of two asexual forms in an infected erythrocyte. Watson (1903) questioned his theory and offered a series

of drawings as proof that the crescent in *Plasmodium falciparum* infections was derived from a young amoeboid parasite and could be differentiated early. Stephens and Christophers (1908) suggested that the gametes developed from forms already present and the differentiation possibly began with the sporozoites. D. Thomson (1911) felt that there was unanimous agreement that the crescents developed from the ordinary "spores" or merozoites of the parasite. From this time on, efforts have been made to discover the stimuli which bring about the formation of the sexual forms.

The following factors have been suggested as stimuli for the development of crescents in *Plasmodium falciparum* infections. Stephens and Christophers (1908), D. Thomson (1911, 1914), MacGilchrist (1915), Thomson and Woodcock (1922), Knowles and Senior-White (1927) offered evidence that crescent formation was due to a development of immunity, or conditions unfavorable to the asexual forms. Conversely, Swellengrebel and Swellengrebel De Graaf (1920), Christophers (1924), Sinton, Bailey and Chand (1926), Kligler and Reitler (1928) showed that crescent formation was reduced when immunity became marked, or that it was greater during the stage of "acute infestation." By culture experiments, Knowles and Senior-White (1930) disagreed with Sinton's (1926) suggestion that alteration of the pH of the environment engendered greater crescent production. The same authors report that in cultures of *Plasmodium vivax* and *Plasmodium falciparum*, the young rings produced either abundant schizonts with few or no gametocytes or abundant gametocytes with few or no schizonts.

Other stimuli suggested from clinical observations on *Plasmodium falciparum* infections are: Insufficient doses of quinine (Thomson, D., 1914), administration of quinine (Green, 1929), seasonal incidence (Sinton, 1926), association with chemico-physiological changes in the blood (Abrami and Senevet, 1917). These views are summarized and discussed in the monograph by Knowles and Senior-White (1930) and, recently, in a review by Thomson and Robertson (1935).

The tendency to regard gametocyte production as a function

of the parasite, irrespective of the influence of the host is relatively recent. Garnham (1931) was the first to call attention to the fact that the apparent increase in crescents might be due to the ordinary waves of crescent production rather than the results of quinine medication. He attempted to stimulate crescent production by the injection of serum from cases with large numbers of crescents into patients in which the infection was just beginning, but obtained negative results.

Boyd (1935) in an extensive study of *Plasmodium vivax*, differentiated by nuclear and cytoplasmic criteria five series of cells, one series being the stem cells for the others, from which arose two series of pregametocytes eventually giving rise to macro- and microgametocytes after 96 hours. In collaboration with Stratman-Thomas and Muench (1936) the same author offered evidence that the process was not a uniformly continuous affair, but rhythmical with upward trends at five-day intervals after completion of a fairly definite number of schizogonous divisions. From experimental inoculations by infected mosquitoes they detected asexual forms as early as the eighth day, but no gametocytes until the fourteenth day, although these were sometimes present along with the first trophozoites and were invariably found at some time during the disease when the illness exceeded 16 days duration. A somewhat similar study was conducted by St. John (1928) on cases of benign tertian malaria induced by direct blood inoculations. He found that the gametocytes were present on the third day of the infection and that the ratio of sexual and asexual forms remained fairly constant from the beginning to the end of the infection.

Since Wagner-Jauregg's introduction of malarial therapy in the treatment of paresis, the subject has been complicated further by the advent of strains apparently lacking in the ability to form gametocytes. Evidence for and against these claims have been discussed in an earlier paper (Huff and Gambrell).

Most of the results obtained in this study tend to substantiate the concept that gametocyte production is an inherent quality of the parasite, rather than a function dependent solely on environmental stimuli. This belief is founded on several observations;

first, that strains vary in their ability to produce gametocytes, but a strain which shows a high percentage of gametocytes usually continues to produce this high number when passed from bird to bird by subinoculations during patent infections or during latency. In several cases where the infection which originally showed a large number of gametocytes has been latent for three years, subinoculations from these birds resulted in infections with a large number of gametocytes. On the other hand, strains which show a small number of gametocytes, or no gametocytes retain these characteristics. The strain with no gametocytes has run true to type through more than a hundred subinoculations, involving countless asexual generations without producing gametocytes. Both of these strains have been in the same environment, so far as it is possible to obtain this condition in experimental animals. Furthermore, no artificial change in the environment, such as disturbing the activity of the host, adding serum from birds infected with the gametocyteless strain, or mixing the two strains caused the strain which regularly produces the full complement of gametocytes to lose its ability to produce gametocytes.

Interpreting the findings in the order presented, it seems evident that gametocytes arise from merozoites, or at the time the asexual forms arise, and increase in definite rhythmic waves. From cytological studies of parasites on synchronous cycles broods of pregametocytes were detected and differentiated from the schizonts when the development of the latter had advanced so far that nuclear division was taking place. From this time on, the young gametocytes could be distinguished by the structure of the nucleus, staining characteristics and arrangement of pigment. The numbers of gametocytes were augmented periodically, apparently at each segmentation period of the asexual parasites. When the time of asexual segmentation was altered by changing the periods of light and darkness, and thereby causing the host to undergo periods of activity and rest, on a schedule exactly opposite to its previous schedule (Boyd, 1929b) then the rhythm of gametocyte production adapted itself to this change also, indicating that the sexual and asexual elements are closely

related, or else influenced by the same factors. Shah (1934) reported the periodic appearance of gametocytes of *P. cathemerium* in the peripheral circulation and these findings amplify his results. In the two species studied here, maturation took longer than 24 hours, requiring from 30 to 32 hours.

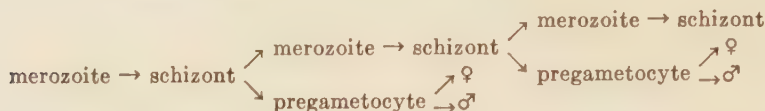
Efforts to go back as far as the merozoites and find in them some distinguishing feature which would differentiate those destined to become sexual forms from those which would carry on the asexual cycle were not successful, in spite of the availability of a strain which lacked gametocytes for comparison with the original strain having the full complement of gametocytes. Any difference which the latter might show and the former might not would be considered as having significance. The most outstanding difference was in the numbers of merozoites in the segmenting forms of these two strains.

Very little is known of the cytogenetic components of *Plasmodium*. The nucleus is referred to as a chromatin mass and studies of its structure, particularly of chromosomes if they be present, must await more refined technique. The small size of the nucleus and the present available methods of staining close the approach of a genetic study based on chromosome behavior. The significance of the definite staining spherical mass within the nucleus of the macrogametocyte and the pregametocyte is unknown, but indicates the great need of further cytological investigations on this group of protozoa. Genetic studies on the plasmodia are of particular interest and importance since their ultimate survival in nature depends on sexual reproduction. As pointed out by Huff (1934) each transmission by mosquitoes hypothetically yields a strain of malarial organisms differing genetically from the original since the parasites undergo sexual reproduction.

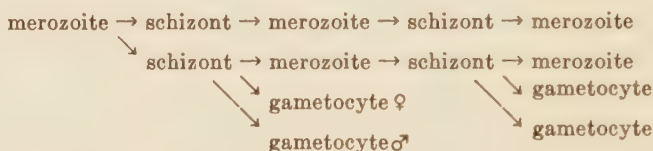
In the absence of morphological evidence of the manner in which gametocytes are differentiated from the asexual forms in their earlier stages, a tentative hypothesis for their mode of development is almost obligatory. Since all our findings do not fit in with the theories already advanced the following hypotheses are offered as a possible explanation of their development.

1. Only one kind of merozoites is produced and these are

capable of becoming schizonts and gametocytes, and these subsequent schizonts possess this same capability ad infinitum. This may be expressed diagrammatically as follows:



2. A merozoite may be produced which gives rise to two potentially different forms one of which produces only asexual parasites, the other producing asexual and sexual forms. This may be expressed diagrammatically in this way:



The first mode of development seems supported by the following facts: First, Stauber in this laboratory (work to be published) obtained an infection from the inoculation of a single cell which showed both the asexual and the sexual forms. Second, inoculations from birds with latent infections in which very few parasites are present give rise to infections with both sexual and asexual forms, and the numbers of the asexual forms increase. Third, in some old laboratory strains which have been passed for years by subinoculation there has been no diminution in the ability to produce gametocytes. When we remember that the production of a gametocyte in malaria is a biological blind alley unless it is picked up by a mosquito, some inexhaustible source of gametocytes must be postulated. Fourth, this would explain the rhythmic appearance of pregametocytes in relation to the asexual cycle. This helps explain why infections do not increase by geometric progression for some of the merozoites are becoming gametocytes at each segmentation, as well as being phagocytosed. The very high percentage of gametocytes seen in some individual birds may be explained on the chance survival of the forms which are to become gametocytes rather than the asexual forms, for it has

been demonstrated by Taliaferro (1925) that only a small percentage of merozoites formed at each schizogony escape phagocytosis and mature.

This mode of development does not account for the appearance of a strain lacking gametocytes, unless it is assumed that some harmful extrinsic factor has damaged the schizont so that the merozoites destined to become pregametocytes are absent in succeeding generations, or that a mutation has taken place which inhibits the expression of the sex characters. In this respect the smaller number of the merozoites in the segmenting form of the strain which lacks gametocytes may have significance. Boyd and Allen (1934) found a wide variation in the number of merozoites produced during the course of infection with *P. cathemerium*. These same authors and Lourie (1934) found that administration of quinine caused a marked decrease in the numbers of merozoites. Although no harmful agent could be demonstrated in infections with the "F" strain either by isolation of the agent or by any effect on the "D" strain when the infections were purposely mixed, its existence cannot be conclusively ruled out, as the parasites may have been permanently damaged while in one host.

The second hypothesis of gametogenesis accounts for a strain which lacks gametocytes but does not account for the development of both sexual and asexual forms from single cell isolation. Further isolations may give rise to other strains lacking gametocytes.

The fact that a cyclo-propagative parasite involving two alternative hosts, a vertebrate and an invertebrate, when transmitted abnormally, i.e., from vertebrate to vertebrate, may eventually lose some of its characteristics is not a biological rarity. Similar changes have been reported for human malaria by Barzilai-Vivaldi and Kauders (1924), Cuboni (1926), Plehn (1924, 1925) and Giovannola (1935); and for *Theileria parva* by Sargent et al. (1932). The surprising thing is that it does not happen more often. A long residence in one host with constant asexual reproduction seems to enhance the asexual characteristics and to diminish the sexual characteristics. Giovannola calls attention

to the fact that long chronic infections of human malarias are characterized by premature segmentation, a small number of merozoites in the segmenting forms and a few or no sexual forms, due to an abnormal situation forced on the parasites.

Certain strains of malaria do vary in their ability to produce gametocytes. Marchoux (1922) stated that *P. falciparum* of the West Coast of Africa forms fewer gametocytes than, and differs morphologically from the same species found in central Italy and Macedonia. Kortweg (1930) in his work with inoculation malaria also found a difference in his strains as to their gametocyte producing ability. In this work the varying ability of different strains to produce gametocytes has been especially noticeable.

This particular study has raised many pertinent questions which need further investigation before a complete answer can be formulated. It involves such fundamental problems as the stimuli to the formation of the sexual elements, and the inhibition of this process which would lead eventually to the destruction of the parasite.

SUMMARY AND CONCLUSIONS

1. Normal gametogenesis in two species of bird malaria, *P. cathemerium* and *P. relictum* var. *matutinum* was studied. Criteria for distinguishing pre-gametocytes and micro- and macro-gametocytes based on cytological characteristics are offered for both species.

2. Gametocyte production in these two species which have a strict quotidian periodicity of the asexual cycle, was found to have a periodicity also. Pregametocytes are more prevalent at the segmentation time of asexual parasites, the numbers are augmented periodically, and new broods are distinguishable. The process of maturation required more than 24 hours, usually 30 to 36 hours.

3. When the time of segmentation of the asexual forms was shifted by changes in the schedule of light and darkness, so that it occurred 12 hours later, the appearance of new broods of gametocytes coincided with the changed segmentation time. When

the synchronism was disturbed by prolonged periods of light and darkness, gametocyte production became irregular.

4. Observations made over a 24 hour period indicated that when mature gametocytes are most plentiful, few gametocytes are found, and vice versa.

5. When gametocytes are mature the ratio of the two sexes is more nearly equal. When gametocytes are prevalent most of them appear to be pre-macrogametocytes but these may be undifferentiated forms.

6. The mean number of merozoites per segmenter in this strain of *P. cathemerium* was $12.95 \pm .17$ in 1935, and $13.72 \pm .16$ in 1936.

7. The initial occurrence of gametocytes in *P. cathemerium* was studied by injections of whole blood, whole blood that was allowed to stand at room temperature, blood meals from mosquitoes and intravenous injections of sporozoites. Gametocytes often appeared simultaneously with the asexual forms, and increased in numbers as the asexual forms increased.

8. Long continued passage by subinoculation tended to decrease the numbers of gametocytes in *P. cathemerium*. Injections from birds whose infections had shown a high percentage of gametocytes and had been latent over a month or more always yielded subsequent infections with a large number of gametocytes.

9. Mosquito transmission for three complete generations of the parasite did not increase the percentage of gametocytes.

10. Further observations were made on a strain of *Plasmodium cathemerium* which does not produce gametocytes. It differs from the parent strain in that it has a "ragged" appearance, lacks pronounced quotidian synchronism and has a smaller number of merozoites per segmenter.

11. The atypical strain failed to show gametocytes when inoculated into its natural host, the English sparrow.

12. Attempts to infect susceptible mosquitoes with the atypical strain and to transfer by mosquitoes were unsuccessful.

13. The atypical strain showed a complete cross immunity to the typical strain and had no inimical effect on the typical strain in mixed infections.

14. Serum from birds with infections of the atypical strain when mixed with parasites of the typical strain had no effect on gametocyte production. Serum from birds with typical infections did not stimulate gametocyte production when mixed with parasites lacking gametocytes.

15. Infection with the atypical strain was still present but latent in two birds after a period of three and one-half years respectively. Subinoculations from these birds resulted in infections that continued to show atypical characteristics.

16. Two birds used in cross immunity experiments were alive after three years, and were reexamined and found to have undergone no changes in the type of infection which they had.

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PLATE 1

Photographs made from stained slides by University of Chicago Photographic Department, Billings Hospital. Magnification 2200 $\times$ unless otherwise indicated.

1. Pregametocyte of "R" strain of *P. relictum*.
2. Erythrocyte infected with 2 pregametocytes, "R" strain.
3. Erythrocyte containing segmenting form and pregametocyte of "R" strain.
4. Mature macrogametocyte of "R" strain.
5. Mature microgametocyte of "R" strain.
- 6 and 7. Pregametocytes of "D" strain of *P. cathe-merium*.
8. Mature macrogametocyte and microgametocyte of "D" strain of *P. cathe-merium*.
- 9 and 10. Microgametocyte and macrogametocyte of "D" strain of *P. cathe-merium*, magnification 3600 $\times$. Karyosome evident in the macrogametocyte.
- 11 and 12. Segmenting form of "F" strain, 1935.
13. Segmenting form of "D" strain in canary, 1936.

